photodetectors, it is possible to measure the amplification of nucleic acid while the reaction is proceeding.

The outstanding rejection is based on a combination of three references. Mullis *et al.* are relied upon for teaching PCR. Sutherland *et al.* are relied upon for disclosing the use of fluorescent dyes such as ethidium bromide for measuring polymerization of nucleic acids. Kaledin *et al.* are relied upon to show that the minimum concentration of ethidium bromide which "inhibits *Thermus flavus* DNA polymerase is 5 μM and that half maximal inhibition requires 23 μM ethidium bromide."

In the previous obviousness rejection, the Examiner viewed Mullis and Sutherland as sufficient to set forth the *prima facie* case of obviousness. The Examiner reasoned that the use of ethidium bromide by Sutherland to measure polymerase activity in a PCR reaction mixture was sufficient motivation to combine ethidium bromide in an actual PCR reaction. In response, applicants urged that the inhibitory effect of ethidium bromide on polymerase activity was sufficient to negate any motivation to combine ethidium bromide in a PCR assay.

To provide proof of the inhibition of polymerization by DNA binding agents, applicants submitted Kaledin, which reports the purification and properties of a *Thermus flavus* DNA polymerase. Among the tests performed by Kaledin was a polymerase activity assay where 160 μg/ml of activated calf thymus DNA was combined in a suitable buffer with the polymerase and dNTPs. The polymerase activity and its sensitivity to ethidium bromide was measured by the incorporation of radiolabelled dTTP.

The remaining obvious rejection is an extension of the previously rebutted rejection. The Examiner further supports her obviousness rejection of the pending claims on the teachings of Kaledin. At table three, Kaledin reports that the reaction was 50% inhibited at 23 μM EtBr and minimally inhibited at 5 μM. Thus, the Examiner apparently reasoned that one of skill reading Mullis, Sutherland, and Kaledin would anticipate that the addition of EtBr at levels of less than 5 μM would not have any inhibitory effect on PCR, and therefore the addition of EtBr to a PCR

reaction at low levels such as disclosed by the applicants would be unpatentably obvious.

Applicants will provide multiple scientifically valid reasons why one of skill would not view Kaledin as motivation to use relatively low amounts of EtBr in a PCR mixture to avoid inhibition. These reasons are restated in a Rule 132 declaration by Dr. Richard Abramson for the purpose of perfecting a suitable evidentiary record. First, it will be explained that the Examiner's interpretation of Kaledin *et al.* is based on a misapprehension of the mechanism of inhibition. The polymerase is not inhibited by ethidium bromide. It is the extension of primer/template complex that is inhibited by EtBr and it is the EtBr:DNA ratio that determines if the polymerase assay will be inhibited, not the ratio of EtBr to enzyme. Furthermore, the DNA binding EtBr in the Kaledin *et al.* assays is mostly, if not entirely, double stranded. While in a PCR reaction, the predominant DNA is single stranded primers. Because EtBr binds primarily to double stranded DNA under the salt conditions of a PCR, any rejection relying on the teachings of Kaledin to suggest that low levels of EtBr would not inhibit PCR would be based on hindsight and speculation - not on any sound scientific reasoning. Furthermore, Dr. Abramson has compared the relative ratios of EtBr:DNA reported in the Kaledin reference to the various examples disclosed in their specification. The analysis reveals that the claimed invention operates without inhibition at ratios reported by Kaledin to be at least 50% inhibitory of a polymerase activity assay.

Finally, it will be explained that the mechanism of EtBr inhibition of 3' extension is such that its inhibitory effects were considered to be of less concern in a polymerase activity assay in contrast to a PCR where each primer extension must be complete in order to have exponential amplification occur, and where duplex denaturation is needed to ensure the availability of all the templates.

The Law.

Prior to articulating in detail the multiple bases for rebutting the Examiner's rejection, applicants would like the Examiner to consider the legal position being presented by the rejection. The Examiner has identified most of the

elements of the invention. PCR was known and EtBr was known to be useful to monitor polymerase activity and Kaledin taught that in their assays, using 5 μM EtBr, inhibition of polymerase extension was minimal. In view of the prior art, the Examiner concludes that it would be obvious to add low amounts of EtBr to a PCR and monitor amplification. In rebuttal, applicants will respond with scientific reasons why the Examiner's conclusion is in error.

The rebuttal of a *prima facie* case of obviousness is a standard response by a patent applicant. Applicants presume the Examiner is familiar with this law and applicants will not restate it in detail. However, because the Examiner's logic is superficially compelling and because applicants' rebuttal relies on a perceptive understanding of the underlying science, applicants would like to remind the Examiner of the words of the 6th Circuit Court of Appeals in Buzzelli v. Minnesota 178 USPQ 260 (1973). In Buzzelli, the trial court held that if the invention was obvious to it then the invention must be obvious to one of skill. The Court of Appeals reversed relying on a quote from Learned Hand who wrote:

> "To judge on our own that this or that new assemblage of old factors was, or was not, 'obvious' is to substitute our ignorance for the acquaintance with the subject of those who were familiar with it." Reiner v. I. Leon Co., Inc., 285 F.2d 501, 504, 128 USPQ 25, 27-28 (2d Cir. 1960), cert. denied, 366 U.S. 929, 129 USPQ 502 (1961).

The above standard is the applicable legal standard. In deference to the above law, applicants would urge that the Examiner reconsider her rejection in view of the following remarks.

A. THE SINGLE STRANDED DNA OF THE PCR REACTION CANNOT BE COMPARED WITH THE DOUBLE STRANDED DNA OF KALEDIN.

The Examiner would urge that the polymerase assays of Kaledin would suggest, with a reasonable expectation of success, that suitably low levels of EtBr could be used in the Mullis PCR to avoid the effects of polymerase inhibition. Above, applicants advance that the polymerase is not being inhibited. The extension reaction is inhibited. Here, applicants will advance that the double stranded DNA used in the Kaledin assays is not sufficiently comparable to the

single stranded DNA used in PCR to provide one of skill with a reasonable expectation of success that EtBr will not inhibit a PCR.

As Dr. Abramson explains in his declaration, the affinity of EtBr to nucleic acid is primarily as an intercalater binding to double stranded DNA. There is only marginal binding to single stranded DNA. Kaledin describes a reaction mixture having 160 μg/ml of activated calf thymus DNA. This is double stranded DNA that has been partially hydrolyzed with DNAse I. The EtBr is highly attracted to the double stranded DNA in the Kaledin assays. But not all the EtBr is inhibiting the polymerase activity.

The EtBr binds in many sites of the DNA which are not participating in the polymerase extension. As Dr. Abramson explains, the polymerase binds to a gap in the double stranded DNA and if it finds that EtBr is present and the duplex distorted, the extension will not occur. Furthermore if nick translation is occurring, the downstream duplexes are distorted by the EtBr and extension again inhibited. Thus, in Kaledin, the relatively high amount of double stranded DNA acts as a sink in which molecules of EtBr are bound but do not inhibit polymerase activity.

In contrast, the predominant DNA in a PCR mixture is single stranded primers. In a PCR mixture, the primers are typically present in nanogram to microgram quantities while the target DNA is present in nanogram or picogram quantities. Dr. Abramson explains that the differences in the ability of EtBr to bind to single and double stranded DNA make impossible any reasonable predictions as to the effect any particular concentration of EtBr in a PCR. The 5 μM concentration of EtBr used by Kaledin to achieve minimal inhibition of 3' extension using 8 μg/reaction activated calf DNA are simply not analogous to a PCR solution where single stranded DNA predominates at considerably lower concentrations (i.e. 100 to 800 ng per reaction).

Even if one of skill were to speculate, the logic would motivate against adding EtBr to a PCR mixture. More specifically, one might consider that the presence of a large amount of double stranded DNA in Kaledin's assays permitted them to use relatively high concentrations of EtBr before inhibition was

detectable. Without the 8 μg of double stranded DNA to tightly bind free EtBr, the Kaledin assays might have been much more sensitive to EtBr. Because PCR mixtures are predominantly single stranded DNA, the above logic would lead one to believe that EtBr might be a particularly potent inhibitor of PCR based amplification.

As Dr. Abramson states in section 8 of his declaration, a final extension of this logical progression leads one of skill to conclude that if the concentration of EtBr in a PCR had to be reduced following the teachings of Kaledin, the calculated levels of EtBr would have rendered the detection of the signal so low that the invention would have little practical use.

In section 8 of his declaration Dr. Abramson has calculated the ratios of EtBr to DNA used in the Kaledin polymerase activity assays and compared them to the ratios used by the applicants. The results clearly demonstrate that the Kaledin reference TEACHES AWAY FROM THE INVENTION. The Kaledin reference teaches that a ratio of 1.4×10^{-4} μmol EtBr : 1 μg DNA/50 μl Rx yields a 50% inhibition of polymerase activity and that a 3.1×10^{-5} μmol EtBr : 1 μg DNA/50 μl Rx provided only minimal inhibition. A superficial interpretation of Kaledin would lead to the conclusion that EtBr concentrations in excess of 5 μM EtBr poisons the polymerase. The correct interpretation is that greater than 3.1×10^{-5} μmol EtBr is needed to bind to 1 μg of double stranded DNA to inhibit 3' polymerase extension.

Yet surprisingly, the applicants' work demonstrates that the ratios of Kaledin when used in a PCR reaction are not inhibitory. Dr. Abramson normalized the ratios of the applicants' examples on pages 22-29 of the specification. The ratio of μmol of EtBr to 1 μg of DNA per reaction ranged from 1.2×10^{-3} to 1.2×10^{-4}.

This explanation leads to the conclusion that Kaledin teaches away from the invention. If those of skill presumed that the EtBr bound equally to both double stranded and single stranded DNA, they would be surprised to learn that their was no inhibition at the EtBr concentrations preferred by the applicants for ease of detection. And if those of skill correctly noted that the actual concentration of double stranded DNA in a starting PCR was orders of magnitude

lower than used by Kaledin, the proportional concentrations of EtBr for a uninhibited PCR as suggested by the Kaledin teaching would be so low that detection of the reaction would be rendered impractical.

B. UNLIKE THE POLYMERASE ACTIVITY ASSAYS OF THE PRIOR ART, PCR WOULD BE EXPECTED TO BE EXQUISITELY SENSITIVE TO ETBR BECAUSE PCR REQUIRES THAT 3' EXTENSIONS GO TO COMPLETION AND THAT EXTENDED DUPLEXES OF DNA FULLY DENATURE DURING THE THERMOCYCLINGS.

Applicants will present two further scientific reasons why the Kaledin *et al.* reference in combination with Mullis and Sutherland fails to motivate one of skill to add EtBr to a PCR at 5 μM or less. These two reasons reflect the significant physical differences between the assays of Kaledin and Sutherland where EtBr is present, and the PCR amplifications of Mullis. In summary, the polymerase assays of Kaledin and Sutherland are single step assays which are fundamentally distinguishable from the multiple steps of PCR. First, it will be explained that PCR requires each primer to be "fully extended" to permit the resultant oligomer to be used as a template in the next cycle. Second, it will be explained that a successful PCR requires the extended primers of the previous extension phase be denatured prior to the next extension and that EtBr stabilizes duplexes to a degree that renders its use in PCR surprising. Because both of these concerns involve steps in the PCR process that are <u>not</u> a part of the Kaledin or Sutherland assays, applicants urge that the combination of prior art fails to render the claimed invention obvious.

(i) Unlike the polymerase assays of the prior art, PCR 3' extensions must go to completion, and thus prior art suggestions to add EtBr to polymerase activity assays at 5 μM would not motivate the addition of EtBr to PCR.

In his declaration at section 10, Dr. Abramson explains that the mechanism for inhibition of polymerase activity depends upon the ability of the

EtBr to bind to double stranded template nucleic acid and to distort the helix structure. This physical distortion stops the polymerase dependent 3' extension of a primer/template combination. The polymerase is not poisoned by the EtBr. The polymerase simply fails to bind to the distorted template/primer duplex and is inactive until it finds an EtBr free template/primer combination and extension can occur. As with any reaction the free and bound EtBr are in equilibrium. A duplex which is distorted at time A may be free of EtBr at time B. In addition to distorting duplexes of primer/templates, the secondary structure of the target templates will be stabilized by EtBr. In the absence of EtBr, hairpins in the template are normally not a significant problem. But, when stabilized by the EtBr, they can prevent full extension of the template. The Sutherland and Kaledin assays only measure extension and not the length of extension. Thus they are relatively insensitive to EtBr.

In contrast, PCR requires that the polymerase dependent extensions go to completion. For without full extension, the primers of the previous cycle cannot function as a template in the next cycle. Thus, as Dr. Abramson explains, PCR is going to be exquisitely sensitive to any compound that inhibits full extension unlike the activity assays of the Kaledin and Sutherland references.

(ii) Unlike the polymerase assays of the prior art, PCR requires that the DNA duplexes of the previous step be fully denatured prior to extension.

In addition to the fact that the prior art assays are less sensitive to EtBr than PCR because of the need for PCR to complete 3' extension, the EtBr effect upon DNA duplex stability is a further concern. In the prior art activity assays, stabilization of DNA duplexes by EtBr is of no concern. In contrast, duplex stabilization is a significant concern in PCR. As the Examiner presumably understands, PCR requires repetitive denaturation of DNA duplexes after each round of primer extension. According to Dr. Abramson, if one adds a helix stabilizing agent to a PCR, the melting temperature (Tm) of the duplexes will increase dramatically, and thus the kinetics of the reaction would be expected to change. It has already been explained by applicants that the inhibition of PCR, by

even a few percent, dramatically impacts on the final amount of product. Thus, the addition of EtBr to a PCR with its ability to stabilize duplexes and render them unavailable during the next cycle is a second reason why Kaledin's use of low amounts of EtBr in a polymerase assay fails to motivate the analogous use of EtBr in PCR with a reasonable expectation of success.

Finally, applicants would ask the Examiner to note that the invention is contrary to conventional wisdom. A recent review article to be published this year expressly states this fact. The review article is entitled *Rapid Cycle DNA Amplification*. It is to be published as Chapter 15 of Polymerase Chain Reaction, Edited by Mullis, Ferre, and Gibbs. A copy is attached as Exhibit 2 of Dr. Abramson's Declaration. At the carryover paragraph on pages 175-176 of the preprint the authors state:

> Ethidium bromide can also be included at concentrations used for staining gels (0.5 μg/ml) without apparent changes in yield or specificity (Fig. 15.2C). This is surprising because of the known influence of ethidium bromide on DNA melting (Maeda et al., 1990). The potential for using ethidium bromide fluorescence during amplification as a monitor of double stranded DNA production is attractive.

Applicants would urge that such a clear and unsolicited statement of nonobviousness by a third party is seldom available to patent applicants. It is urged that this statement by itself is adequate to rebut the *prima facie* case of obviousness.

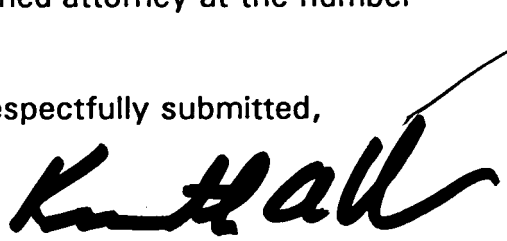
With the above two explanations, applicants believe that the rejection is fully rebutted. Clearly, successful PCR amplifications rely on more than the rate of 3' extension. Unlike the prior art polymerase assays of Kaledin and Sutherland, the PCR 3' extensions must go to completion and the products of extension must be repetitively denatured. With the above understanding of the mechanism of EtBr inhibition of polymerase reactions, it should be clear that one of skill in PCR would not view Kaledin as motivating the use of low levels of EtBr in the PCR reaction mixtures of Mullis. Moreover, even if the combination of references were viewed as motivation to try the claimed combination, the above reasoning is applicable and

would obviate any reasonable expectation of success that one of skill might have harbored. Having explained why both the motivation and expectation of success are not present in the cited combination of references, applicants would urge that the rejection be withdrawn.

In view of the above remarks, applicants request reconsideration of the outstanding *prima facie* case of obviousness of claims 1-22. Applicants have presented multiple scientific explanations why one of skill would not have read the combination of references and have been motivated to combine EtBr with PCR and that even if motivated they would have had no valid reason to conclude that the addition of EtBr at the concentrations suggested by the prior art would not have inhibited PCR. Each of these reasons is individually sufficient to rebut the *prima facie* case of obviousness. In view of the combination of arguments, and with the support of Dr. Abramson's declaration, applicants urge that the rejection is fully rebutted and should be withdrawn.

Applicants believe the claims to be in condition for allowance and respectfully request that the Examiner issue a Notice of Allowance. If the Examiner believes that prosecution might be expedited by a telephone conversation, she is invited to call the undersigned attorney at the number provided.

Respectfully submitted,



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